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The Role of Basement Membrane in Intestinal Barrier to Absorption of Macromolecules and Nanoparticles

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Abstract

Full understanding of the barrier property of mucosal tissues is imperative for development of successful mucosal drug delivery strategies, particularly for biologics and nanomedicines. The contribution of the mucosal basement membrane (BM) to this barrier is currently not fully appreciated. This work examined the role of the BM as a barrier to intestinal absorption of model macromolecules (5 kDa and 10 kDa dextrans) and 100 nm polystyrene nanoparticles. Dextrans and nanoparticles were applied either directly to BM-coated inserts or to an intestinal model, namely differentiated intestinal epithelial monolayers (Caco-2) cultured on BM-modified inserts. The work shows that the BM per se does not impact the diffusion of dextran macromolecules, but severely hinders the movement of nanoparticles. However, importantly, Caco-2 monolayers cultured on BM-coated inserts, which show a remarkably different morphology, display a significantly larger barrier to the translocation of one dextran, as well as nanoparticle systems compared to cells cultured on unmodified inserts. Therefore, this work shows that in addition to presenting a direct physical barrier to the movement of nanoparticles, the BM also exerts an indirect barrier effect, likely due to its influence on epithelial cell physiology. This work is important as it highlights the currently unmet need to consider and further study the barrier properties of the BM in mucosal delivery of biologics and nanomedicines.

Key words: Basement membrane, Biologics delivery, Caco-2 cells, Intestinal absorption, Nanomedicine

Introduction

Biologics and nanomedicines as complex therapeutics are currently almost exclusively administered by injection. Non-invasive delivery is preferred over injections due to patient convenience, elimination of injection-associated side effects and, potentially, reduced costs (1, 2). While mucosal administration is currently not a viable option for systemic delivery of most of these advanced therapeutics – mainly due to the challenge of overcoming the mucosal barriers – research into this field is progressing at a fast pace. This is fuelled by the proliferation and wider clinical use of these therapeutics, as well as advances in drug delivery technologies. Development of successful drug delivery technologies enabling mucosal administration of biologics and nanomedicines for systemic effect relies on comprehensive understanding of mucosal barriers.

Mucosal tissues are structurally organised so to serve the important role of a barrier to systemic entry of material from the external environment. The multiple constituent elements of mucosal tissues that present barriers to drug delivery include mucus, enzymes, epithelium and the basement membrane (BM). The epithelium is considered to be the principal mucosal barrier component to systemic absorption of complex therapeutics such as macromolecules nanomedicines. Mucus has also been shown to hinder the diffusion of some macromolecules and many nanoparticles (3). However, effective drug delivery strategies now exist to promote the diffusion of nanomaterials in mucus (e.g. via the 'PEGylation' approach) (4-7) and also facilitate their translocation across the epithelial barrier (e.g. by exploiting epithelial transcytosis to shuttle material across the epithelium) (8, 9). On the other hand, the BM barrier has not been fully characterised. With an increasing proliferation of biologics and nanomedicines and efforts to achieve non-invasive delivery of these therapeutics, there is a need to characterise the barrier properties of the BM to systemic absorption of these therapeutics across mucosal surfaces.

BMs are thin, specialised sheets of extracellular matrices found between epithelia and connective tissue in the human body (10, 11). Collagen is the main protein of the extracellular matrix (ECM) and is linked by multiple bonds, including disulphide and hydrogen bonding, that gives tensile strength to BM (12, 13). Alongside collagen, laminin, which strongly associates to the cell surface, provides additional organised structural support to the BM (14). BMs have several roles including the regulation of cell adhesion, differentiation and motility (15). BM also serves a filter function due to its selective passage of molecules across its barrier (16).

In this work, we characterised the BM barrier by probing its effect in isolation as a direct barrier to the diffusion of model macromolecules and nanoparticles. Importantly, we also incorporated the BM in a commonly utilised cell culture model of the intestinal epithelium (Caco-2 monolayers) and studied the barrier property of the resulting system in order to ascertain whether the BM may have additional, indirect barrier effects by potentially influencing epithelial cell physiology.

Materials and Methods

Basement membrane extract (BME, commercially known as Cultrex® Pathclear®) was purchased from Trevigen (Gaithersburg, MD). Hank's Balanced Salt Solution (HBSS) modified with sodium bicarbonate, without phenol red and fluorescein isothiocyanate (FITC)-labelled dextrans of average molecular weights of 4 and 10 kDa (FD4 and FD10, respectively) were purchased from Sigma-Aldrich (Poole, UK). Transwell® polycarbonate permeable inserts of 12 mm diameter and 0.4 μm pore size and black 96-well assay plates were purchased from Corning (Corning, NY). Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) and used between passages 64-73. Dulbecco's Modified Eagles Medium (DMEM) with 4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate, trypsin/EDTA solution, antibiotic/antimycotic solution (penicillin, streptomycin and amphotericin) and foetal bovine serum (FBS, non-USA origin) were all obtained from Sigma-Aldrich (Poole, UK). DMEM was supplemented with 10% v/v Foetal Bovine Serum (FBS)

and 1% v/v antibiotic/antimycotic solution. Fluorescent sulfate- and amine-modified polystyrene nanoparticles of 100 nm diameter were also purchased from Sigma-Aldrich (Poole, UK). For immunostaining analysis, paraformaldehyde, Triton X-100, bovine serum albumin (BSA) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (Poole, UK). Anti-human zonula occludens rabbit antibody (ZO-1) was obtained from Zymed, Cambridge Bio Science (Cambridge, UK). Alexa Fluor 488 chicken anti-rabbit IgG was purchased from ThermoFisher Scientific (Loughborough, UK).

Preparation of BM-coated inserts

Transwell inserts were coated with BME following the manufacturer's protocol for the 'thin layer method'. Briefly, BME was thawed and mixed by slowly pipetting the solution up and down, avoiding the introduction of air bubbles. Freshly-thawed BME was then diluted in cold HBSS to achieve a protein concentration of approximately 150 µg/ml. 330 µl of this solution (300 µl/cm^2) was applied to the insert and allowed to incubate at room temperature for one hour. Excess BME was removed, leaving a thin layer of material on the inserts. Coated surfaces were not allowed to dry out, with diffusion studies or cell plating performed immediately after the coating procedure.

Permeability of dextrans and nanoparticles across BM

Freshly prepared, BM-coated Transwell® inserts were utilised for these studies, with uncoated inserts serving as a control. HBSS (1.5 mL), pre-warmed to 37°C, was placed in the acceptor (basal) compartment and 500 µl of dextran solutions or nanoparticle suspensions in HBSS were added to the donor (apical) compartments. FD4, FD10 and nanoparticles were applied at 100 µg/ml. Basal solution (100 µl) was sampled at 20-minute intervals for two hours, with the replacement of sampled solutions with fresh HBSS. Dextran and nanoparticle permeabilities were quantified by fluorescence.

Cell culture

Caco-2 cells were routinely cultured on T75 flasks using DMEM. Cells were seeded on unmodified or BM-coated inserts at 10^5 cells/cm^2. Culture medium was replaced three times per week. Caco-2 cells were typically cultured on inserts for 21–23 days, prior to their use in permeability studies, with measurement of transepithelial electrical resistance (TEER), using a Millicell ERS-2 voltohmmeter obtained from Millipore (Burlington, MA), to confirm cell monolayer integrity.

Permeability of dextrans and nanoparticles across BM-cultured Caco-2 monolayers

Caco-2 cells were cultured on inserts coated with BM, following the methodologies described above. After a minimum 21-day culture, cell monolayer TEER was measured to confirm epithelial barrier integrity and suitability for permeability studies. Culture medium was replaced with HBSS (pre-warmed to 37°C), which was used as a transport medium in these studies; cells were equilibrated in HBSS for approximately 45 min. Dextrans and nanoparticles were applied to the apical side of cell monolayers at 100 µg/ml in HBSS. Basal solution (100 µl) was sampled every 20-minutes for two hours, with sampled solutions replaced with fresh HBSS. Apical-to-basal permeability of dextrans and nanoparticles was quantified by fluorescence.

Effect of BM culture on cell monolayer TEER

Caco-2 cells were seeded on inserts that were freshly coated with BM (as described above) or uncoated inserts. TEER was measured at regular intervals during a 21-day culture for both conditions.

Calculation of apparent permeability coefficient (P_{app})

The permeability of FITC-dextrans and nanoparticles is expressed as the apparent permeability coefficient (P_{app}), calculated using this equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t}\right) x \left(\frac{1}{AxC_0}\right)$$

where P_{app} = apparent permeability in cm/s, ΔQ/Δt is the permeability rate (amount of material permeating over time), A is the diffusion area of the insert or cell monolayer (cm^2) and C_o is the initially applied material concentration.

Cell immunostaining for zonula occludens-1 (ZO-1)

Polarized Caco-2 monolayers were washed with PBS and fixed with paraformaldehyde (4% w/v) on ice for 20 min. Cells were washed with PBS again and permeabilized with Triton X-100 (0.05% v/v in PBS) for 5 min. Cells were re-washed with PBS, followed by washing with BLOTTO (5% instant non-fat dry milk in 10 mM PBS), and subsequently incubated overnight at 4 °C with rabbit, anti-human zonula occludens-1 (ZO-1) diluted in 1% w/v BSA/BLOTTO to 12 µg/ml. Cells were then washed extensively with PBS and treated with AlexaFluor 488-labelled chicken, anti-rabbit IgG (diluted in 1% w/v BSA/BLOTTO to a final concentration of 10 µg/ml) for 1 h at room temperature. Following this, cell monolayers were washed and filters excised and mounted on a microscope slide using a 4',6-diamidino-2-phenylindole (DAPI)-containing ProLong® Gold antifade reagent. Slides were stored at 4°C and imaged within a week using a Nikon Eclipse Ti-E inverted confocal microscope (Nikon Instruments, UK).

Effect of BM culture on cell size

Freshly prepared, BM-coated Transwell® inserts were utilised for this study, with uncoated inserts serving as a control. Caco-2 cells were cultured on inserts coated with BM, following the method described above. After a minimum 21-day culture, cell monolayer TEER was measured to confirm epithelial barrier integrity and suitability for permeability studies. Culture medium was removed and cells were washed with PBS. Following this, PBS was replaced with trypsin/EDTA solution and incubated for 10 min at 37 °C. Caco-2 cells were detached from the polycarbonate membrane by gently tapping the inserts. Trypsin/EDTA solution was diluted with complete media and separated from the cells by centrifugation at 1,500 rpm for 5 min. Cells were re-suspended in media. Caco-2 cells were mixed with tryphan blue (1:1) prior to counting on an automated cell counter (Countess II, Life Technologies, UK).

Statistical analysis

Student's *t*-test was performed for comparisons of two group means, while one way analysis of variance (ANOVA) was utilised for comparison of three or more group means. Experiments were conducted in triplicates and repeated. *P* value of <0.05 was considered statistically significant. ** and * indicate $p < 0.01$ and $p < 0.05$, respectively, whereas "ns" indicates nonsignificant. Statistical analysis was conducted using GraphPad Prism® Software.

Results

Permeability of dextrans and nanoparticles across BM

Permeation of FD4 and FD10 across BM-coated inserts is shown in Figure 1 (A and B, respectively). The data demonstrate that the permeability of both dextrans is not suppressed by BM coating of cell culture inserts (permeability was somewhat reduced but this was not statistically significant in both cases).

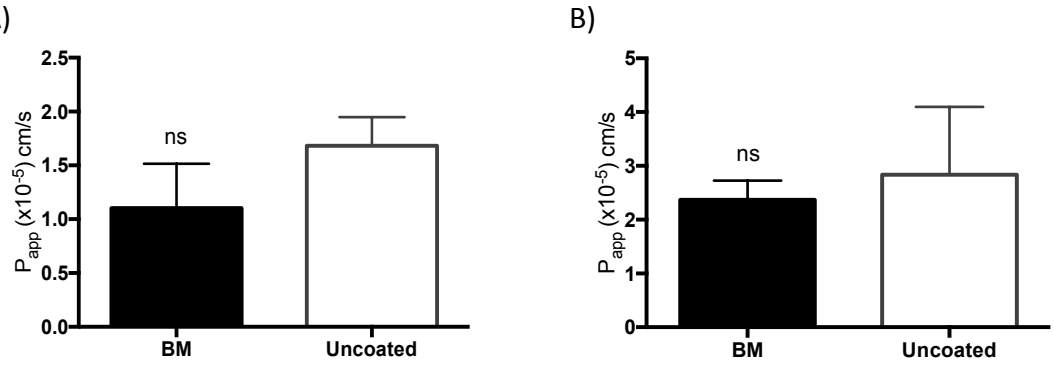

Figure 1. Dextran permeability across basement membrane. Apparent permeability (P_{app}) of dextran macromolecules of 4 kDa (FD4) (A) and 10 kDa (FD10) (B). Basement membrane-coated inserts denoted as 'BM', while unmodified inserts denoted as 'uncoated'. FD4 and FD10 were applied in Hank's Balanced Salt Solution (HBSS) at concentration 100 µg/ml. Data shown as the mean ± SD (n=3).

Nanoparticle translocation across BM-coated inserts is shown in Figure 2. The data clearly demonstrate that BM-coating produces a notable reduction in the permeability of nanoparticles, by a factor of 2.6 and 2.3, for negatively- and positively-charged systems (2A and 2B), respectively. This therefore suggests that BM acts as a barrier to the translocation of 100 nm-sized, negatively-charged and positively-charged nanoparticles.

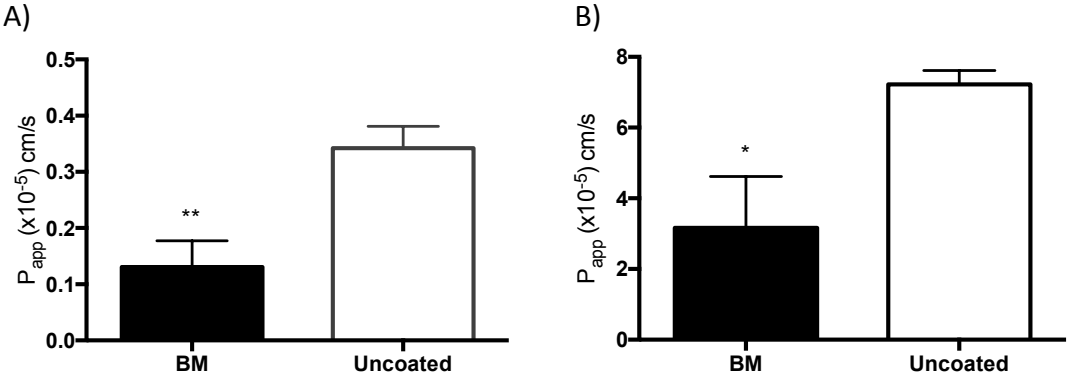

Figure 2. Nanoparticle permeation across basement membrane. A) Negatively-charged 100 nm polystyrene nanoparticles. B) Positively-charged (amine-modified) 100 nm polystyrene nanoparticles. Basement membrane-coated inserts denoted as 'BM', while unmodified inserts denoted as 'uncoated'. Nanoparticles were applied in Hank's Balanced Salt Solution (HBSS) at concentration of 100 µg/ml. Data shown as the mean ± SD (n=3).

Permeability of dextrans across BM-cultured Caco-2 monolayers

The barrier properties of Caco-2 monolayers, cultured on BM-coated inserts, with respect to the permeation of FD4 and FD10 are shown in Figure 3. The data show that FD4 permeability is significantly reduced in BM-cultured cell monolayers compared to that across cells cultured on uncoated inserts (Figure 3A). This pattern however was not apparent for FD10, where culture of cells on BM-coated inserts did not have an effect on the extent of the barrier that the resulting epithelial monolayers present to this molecule (Figure 3B).

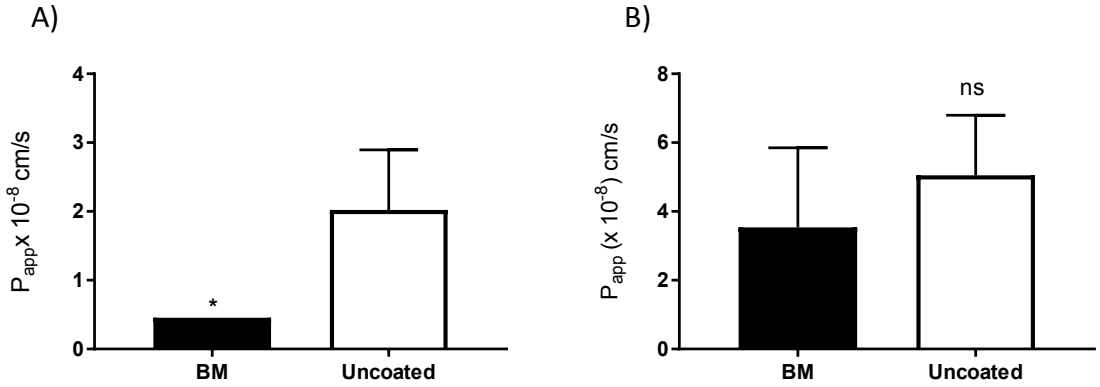

Figure 3. Effect of basement membrane culture on Caco-2 monolayer barrier to dextrans. A) Permeability of fluorescein isothiocyanate (FITC)-labelled dextran of average molecular weight 4 kDa (FD4). B) Permeability of FITC-labelled dextran of average molecular weight 10 kDa (FD10). Basement membrane-coated inserts denoted as 'BM', while unmodified inserts denoted as 'uncoated'. FDs were applied in Hank's Balanced Salt Solution (HBSS) at concentration 100 µg/ml. Data shown as the mean ± SD (n=3).

Permeability of nanoparticles across BM-cultured Caco-2 monolayers

Work examining the barrier capacity of BM-cultured Caco-2 cells towards the permeability of nanoparticles revealed that cell monolayers cultured in this manner markedly hindered the translocation of model nanoparticles (sulfate-modified systems were used in this experiment) markedly more than Caco-2 monolayers cultured on uncoated inserts (Figure 4). Specifically, nanoparticle translocation was approximately four times lower across intestinal epithelial Caco-2 monolayers cultured in the presence of BM.

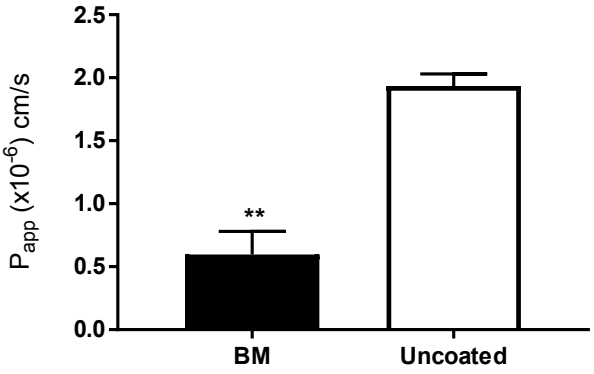

Figure 4. Effect of basement membrane culture on the Caco-2 monolayer barrier to nanoparticles. Sulfate-modified polystyrene nanoparticles of 100 nm diameter were used. Basement membrane-coated inserts denoted as 'BM', while unmodified inserts denoted as 'uncoated'. Nanoparticles were applied in Hank's Balanced Salt Solution (HBSS) at concentration of 100 µg/ml. Data shown as the mean ± SD (n=3).

Effect of BM culture on cell monolayer TEER

To better understand the effect of BM on the influence of the intestinal epithelial Caco-2 barrier, we conducted regular TEER measurements of cells cultured on BM-coated inserts throughout a 21-day culture period. The data in Figure 5 highlight that BM-coating had a significant impact on cell monolayer TEER. Notably higher TEER values in BM-cultured cells compared to cells grown on unmodified inserts were apparent throughout the entire culture period (all measurement points).

This includes the early phase of culture with steep increase in TEER with time (up to day 10), as well as at a later phase of TEER plateau, which is typically seen with Caco-2 monolayer culture.

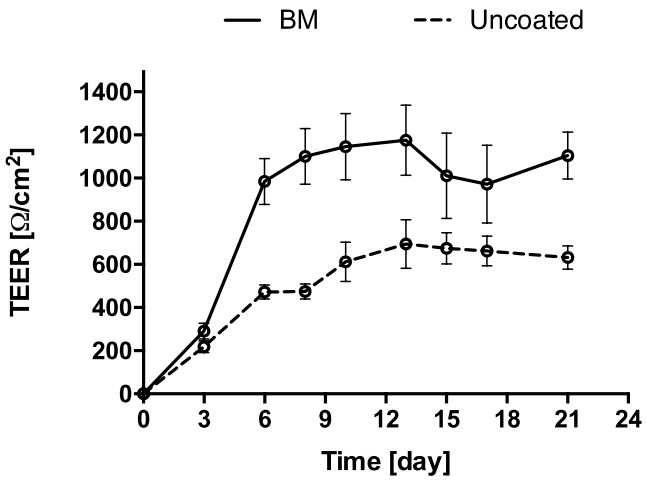

Figure 5. Effect of basement membrane on Caco-2 monolayer transepithelial electrical resistance (TEER). Cells were seeded on basement membrane coated inserts ('BM') or unmodified inserts ('uncoated'). TEER was measured in culture medium at days 3, 6, 8, 10, 13, 15, 17, and 21. Data shown as the mean ± SD (n=12).

Effect of BM culture on zonula occludens-1 (ZO-1)

As ZO-1 is a critical regulator of epithelial tight junctions and the resulting epithelial barrier and the BM showed clear effects on the Caco-2 monolayer barrier, we examined the influence of BM culture on Caco-2 ZO-1 protein expression and localisation. Figure 6 shows confocal micrographs of polarised Caco-2 cells grown on BM-coated (A) and uncoated (B) inserts. The data in Figure 6 reveal significant differences between the two conditions in terms of cell monolayer appearance. Caco-2 cells cultured on BM-coated inserts appear to be significantly bigger, therefore occupying a larger area compared to cells grown on uncoated inserts. This is clearly apparent by the significantly lower number of cells (per equivalent area) that appear more spread out and with dramatically larger ZO-1 staining circumference (typical 'chicken wire' appearance) on BM coated inserts (A), compared to the larger number of smaller cells, suggested by smaller ZO-1 perimeter, on uncoated inserts (B).

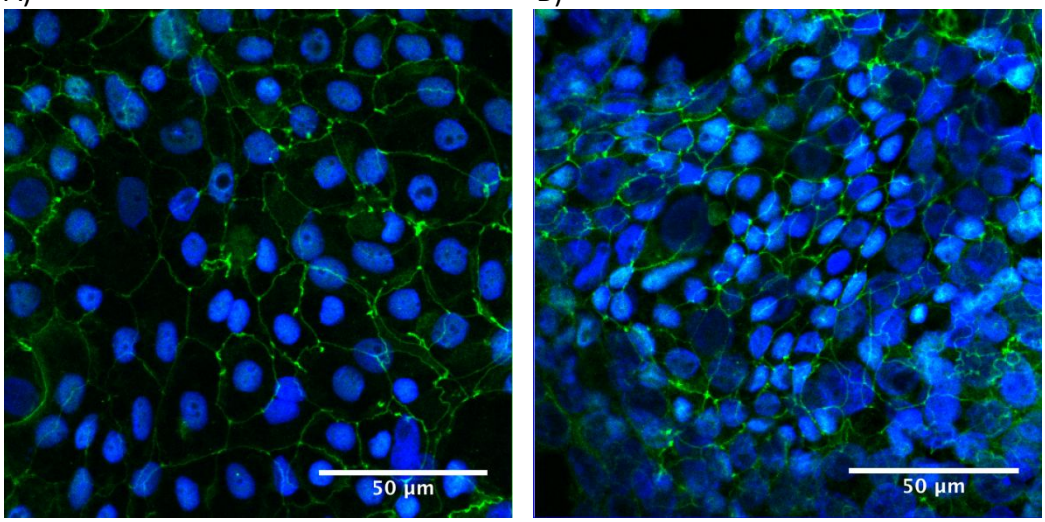

Figure 6. Caco-2 immunostaining for zonula occludens-1 (ZO-1) tight junction protein. Caco-2 cells were cultured for 21 days. Cells were incubated with rabbit, anti-human ZO-1 (primary) antibody, followed by AlexaFluor 488 chicken, anti-rabbit IgG (secondary antibody). Blue channel: cell nuclei stained with DAPI (4',6-diamidino-2-phenylindole); green channel: AlexaFluor (ZO-1 staining) fluorescence. A) Caco-2 monolayers cultured on BM-coated inserts. B) Caco-2 monolayers cultured on uncoated inserts.

Effect of BM culture on cell size

The difference in cell size between culture conditions employing BM and those without, as noted in confocal micrographs (Figure 6) was tested quantitatively. Data in Figure 7 show an average Caco-2 cell size of 11.21 µm and 9.84 µm following culture on BM-coated (A) and uncoated inserts (B), respectively.

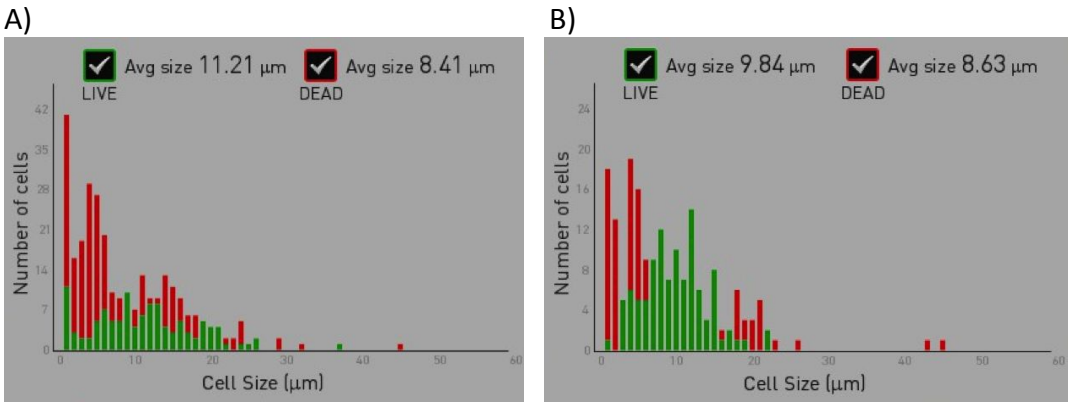

Figure 7. Effect of basement membrane (BM) culture on cell size. A) Caco-2 monolayers cultured on BM-coated inserts. B) Caco-2 monolayers cultured on uncoated inserts.

Discussion

Mucosally-administered drugs for systemic effect require satisfactory absorption from mucosal surfaces. This process also involves drug diffusion through the interstitum (after traversing the epithelium) into systemic circulation via capillary or lymphatic uptake. Factors that dictate mucosal (e.g. intestinal) absorption of small drug molecules are well established and have informed drug development (physicochemical requirements of drug molecules) for decades.

There are a number of physicochemical barriers operating at mucosal absorptive sites that potentially hinder drug absorption. While it is widely accepted that, in the case of intestinal drug absorption, the epithelium with its closely packed polarised cell structure is the key barrier and the limiting factor dictating systemic absorption of drug molecules, there is a need to characterise additional, non-epithelial mucosal barrier components. This is particularly the case with complex biotherapeutics and nanomedicines destined for mucosal administration because non-epithelial barriers may affect the systemic absorption of these therapeutics. As the clinical use of biologics and nanomedicines becomes more widespread, it is imperative that the mucosal barriers are fully understood to facilitate the development of successful drug delivery technologies for non-invasive (mucosal) administration of these therapies.

While the diffusion of biomacromolecules and nanomedicines in the mucus barrier has been studied and strategies to overcome this barrier described (7, 17), it is currently unclear whether the BM, as a structure underneath the epithelium that must also be overcome for systemic absorption, hinders the diffusion of these materials. BM is specialised ECM and the latter has been shown to present a barrier to nanomaterials accessing the tumour compartment (18). For example, particles much smaller than the mesh size of ECM have been demonstrated to be immobilized in the ECM biopolymer matrix (19). This has significant implications for the design and fabrication of nanomedicines for mucosal delivery.

We were first interested to study the barrier behaviour of the BM towards macromolecule diffusion. The data show that the BM *per se* did not hinder the diffusion of FD4 and FD10 (Figure 1) but decreased that of nanoparticles (Figure 2). These findings corroborate with studies in the area. For example, lining of a HeLa monolayer with BM followed by exposure to HPV-16 pseudoviruses reduced the percentage of infected HeLa cells by about 6-fold, highlighting the extent of the barrier that the BM presents to the movement of a 50 nm virus (20).

While studies on the barrier properties of BMs of mucosal tissues relevant to drug administration are limited, much more is known about material diffusion in complex 3D biological environments, including ECM (21-23). It is appreciated that the complex ECM environment presents a major barrier that potentially limits the therapeutic success of nanomedicines (23). The mobility of the materials diffusing in ECM is restricted by both its pores and electrostatic interactions with the network-forming ECM components, hence filtering process consist of both size and interaction filtering (23). Therefore, the mobility of diffusing particles in ECM is chiefly affected by hydrodynamic diameter and surface charge. In terms of interaction filtering, particle diffusion through the ECM is influenced by complex interaction via electrostatic and hydrophobic interactions. Although a net negative charge predominates (from sulphate- and carboxyl-rich glycosaminoglycans), ECM matrix carries both positively and negatively charged components. It is thought that the composition and complex cross-links between ECM macromolecular components are both critical for the filtering process (23). ECM was shown to dramatically suppress the diffusion of both positively and negatively charged particles that were significantly smaller than its mesh size (19).

Our results derived from the studies incorporating BM into Caco-2 culture are interesting. The Caco-2 intestinal in vitro model is based on cell culture directly on plastic porous substrates and lacks the ability to produce a BM (24, 25). However, the intestinal epithelium in vivo is supported by a BM, which not only plays an essential role in controlling a variety of epithelial phenomena – including cell attachment, growth, migration and differentiation (26-28) – but can also affect the mucosal barrier. This effect can arise directly by the BM acting as a physical barrier towards the diffusion of material into the subepithelial interstitium, as well as indirectly, through a BM effect on the morphology and physiology of the epithelium it supports.

We observed that the BM significantly altered the barrier properties of Caco-2 monolayers to one of the tested dextrans (Figure 3), as well as nanoparticles (Figure 4). It is interesting that while FD4 permeability was reduced notably (by approximately a factor of four), no effect was observed for FD10. The mechanism behind this observation could be related to a BM effect on the paracellular permeability, with a larger proportion of FD4 more likely to traverse the epithelial monolayers by this route, as opposed to FD10 (29). Hence, any effect on the paracellular pathway barrier will show a larger influence on FD4 permeability.

To further probe the influence of BM on the epithelial paracellular barrier, we studied the TEER *vs* time profile, as well as the morphology of paracellular barrier-controlling tight junctions of Caco-2 cells cultured on BM-coated inserts. In terms of TEER, the data in Figure 5 demonstrate clearly that the presence of BM leads to Caco-2 monolayers with a more restrictive paracellular barrier (higher TEER throughout the culture period). Staining for the tight junction protein, ZO-1, revealed a striking difference in cell morphology, with BM-cultured cells appearing larger in size (Figure 6). This was also confirmed quantitatively via cell size measurements (Figure 7).

The indirect effect of the BM on the epithelial barrier is unsurprising given that previous studies have shown that focal adhesions, formed at cell-matrix contact points, influence the development and maintenance of tight junctions via the actin cytoskeleton (30-33). There is established evidence that the interaction of epithelial cells with the BM via cell surface integrins influences the expression of TJ proteins (34, 35). Our findings related to the BM effect on cell morphology may explain the barrier properties of Caco-2 epithelial monolayers. Specifically, a polarised monolayer with larger epithelial cells, such as one cultured on BM-coated inserts, will have a smaller paracellular surface area compared to a monolayer with smaller cells cultured on the same substrate area. This may therefore explain the finding that FD4 but not FD10 (i.e. the compound more likely to utilise the paracellular route) displayed attenuated permeability across BM-cultured Caco-2 monolayers compared to control.

Overall, this work demonstrates that the BM plays an important role as a barrier to mucosal delivery of macromolecules and nanomedicines. This barrier-influencing role of the BM arises via a direct effect of BM as a physical barrier to the diffusion of materials, likely to be the case for nanoparticle diffusion, or by an indirect effect on epithelial physiology (more likely to impact macromolecule absorption).

Conclusion

This study shows that BM acts as a drug delivery barrier for intestinal delivery of macromolecules and nanomedicines in different ways. Strategies for mucosal delivery of complex therapeutics should therefore address the BM, in addition to other mucosal tissue barrier components. Furthermore, research employing in vitro mucosal models to study and predict mucosal delivery of macromolecules and nanomedicines should consider the incorporation and recapitulation of this important mucosal barrier component to improve the predictive value of the model.

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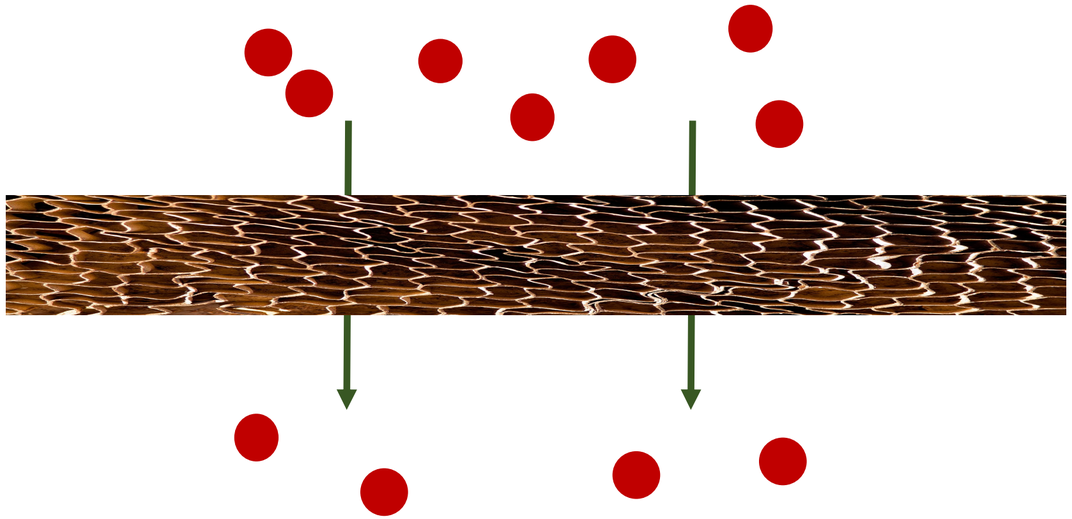
Basement Membrane:

1. Direct Barrier to Nanoparticle Permeability



2. Effect on Cell Morphology & Paracellular Permeability